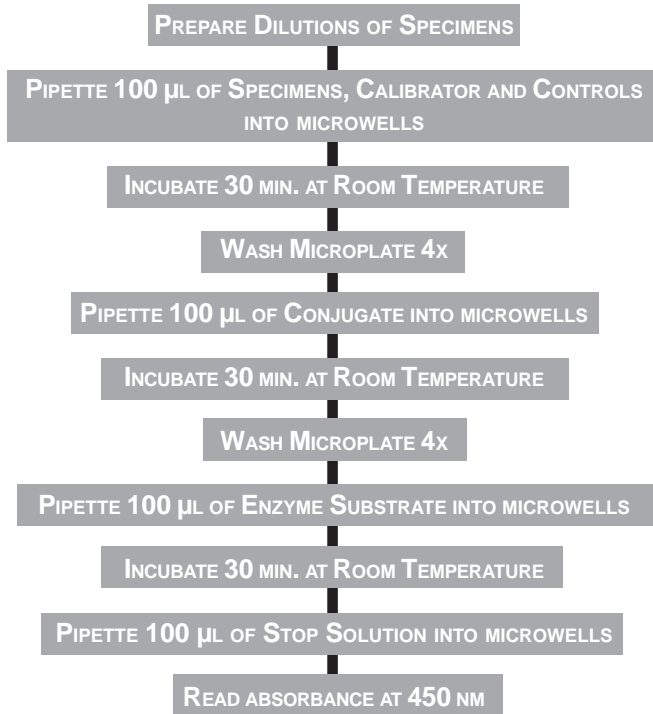


Immulin™ PROCEDURE AT A GLANCE



For technical assistance please contact:



IMMCO Diagnostics, Inc.

60 Pineview Drive
Buffalo, NY 14228-2120

Telephone: (716) 691-0091

Fax: (716) 691-0466



Toll Free USA/Canada: 1-800-537-TEST

E-Mail: info@immcodiagnostics.com

or your local product distributor



EU Authorized Representative/Autorisierter Repräsentant/Rappresentante
Autorizzato/Representante Autorizado/Représentant Autorisé
EMERGO Group, Inc.

Molenstraat 15, 2513 BH, The Hague,
The Netherlands

Tel (+31) 345 8570, Fax (+31) 346 7299
www.emergogroup.com



Immulin™ Anti-Nuclear Antibody (ANA) Screen ELISA

For *in vitro* diagnostic use

Code 1175

PRODUCT INSERT

96 Determinations

INTENDED USE

An enzyme linked immunosorbent assay (ELISA) for the detection of antinuclear and cytoplasmic antibodies in human serum to aid in the diagnosis of autoimmune diseases such as Systemic Lupus Erythematosus (SLE), Sjögren's Syndrome (SS), Mixed Connective Tissue Disease (MCTD), and Scleroderma.

SUMMARY AND EXPLANATION

Anti-nuclear antibodies (ANA) are a group of antibodies directed against various nuclear and some cytoplasmic antigens. Serological tests for ANA play an important role towards the diagnosis of various autoimmune connective tissue disorders especially systemic lupus erythematosus (SLE), scleroderma, mixed connective tissue disorder (MCTD) and sjogren's syndrome¹⁻⁵. ANA are usually detected by indirect immunofluorescence on Hep2⁶⁻¹⁰. Because of certain limitations of IFA, a need for non-subjective method of detecting ANA has been realized. The enzyme immunoassay (ELISA) offer several advantages over IFA method of detecting ANA such as ease of operation and not requiring skills needed to perform and read IFA reactions¹¹⁻¹⁵. As the ANA are sensitive but not specific indicators of a connective tissue disease, it is recommended that more specific antibody tests be performed in patients with positive ANA suspected of having an autoimmune connective tissue disorder. Also it is suggested that positive ANA results on ELISA may be confirmed by indirect IF on Hep2 as this may also help to identify the reaction pattern of the ANA reaction which has significance¹⁶.

PRINCIPLES OF PROCEDURES

The ANA test is performed as a solid phase immunoassay (ELISA). Microwells of the microplate are coated with antigens from the Hep2 supplemented with other nuclear and cytoplasmic antigens followed by blocking the unreacted sites to reduce nonspecific binding. Controls, calibrator and patient serum samples are incubated in the antigen coated wells which allows ANA present in the serum to bind to the adsorbed antigen on the microwells. Unbound antibody and other serum proteins are removed by washing the microwells. Antibodies bound to the microwells are detected by adding enzyme labeled anti-human IgG conjugates to the wells. These enzyme conjugated antibodies bind specifically to the ANA bound to the antigen coated wells. Unbound enzyme conjugate is removed by washing. Specific enzyme substrate (TMB) is then added to the wells and the presence of ANA are detected by a color change produced by the conversion of the substrate to a color product. The reaction is stopped by the addition of stop solution and the intensity of color change, which is proportional to the concentration of antibody, is read by a spectrophotometer at 450nm. Results are expressed in ELISA units per milliliter (EU/ml).

REAGENTS

Storage and Preparation

Store all reagents at 2-8°C. **Do not freeze.** Do not use if reagent is not clear or if a precipitate is present. All reagents must be brought to room temperature (20-25°C) prior to use. When stored at 2-8°C, the reconstituted wash buffer is stable until the kit expiration date. Reconstitute the wash buffer to 1 liter with distilled or deionized water. Coated microwell strips are for one time use only.

Precautions

For *in vitro* diagnostic use. All human derived components used have been tested for HBsAg, HCV, HIV-1 and 2 and HTLV-I and found negative by FDA licensed tests. However, human blood derivatives and patient specimens should always be considered potentially infectious. Follow good laboratory practices in storing, dispensing and disposing of these materials¹⁷.

WARNING - Sodium azide (NaN₃) may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal of liquids, flush with large volumes of water to prevent azide buildup. Sodium azide may be toxic if ingested. If ingested, report incident immediately to laboratory director or poison control center.

Dispose of reagent solutions containing sodium azide and Proclin as preservatives according to all local, state and national regulations.

Instructions should be followed exactly as they appear in this kit insert to ensure valid results. Do not interchange kit components with those from other sources other than the same lot number from IMMCO Diagnostics. Follow good laboratory practices to minimize microbial and cross contamination of reagents when handling. Do not use beyond expiration date on the label.

Materials Provided

Kit contains sufficient reagents to perform 96 determinations each.

- 12 x 8** Ready to use **Microplate** with individual breakaway microwells coated with anti-nuclear antigen.
 - 1 x 1.5 ml** Ready to use **Positive Control*** (*red cap*). Contains human serum positive for IgG ANA. The expected concentration range in EU/ml is printed on the label.
 - 1 x 1.5 ml** Ready to use **Negative Control*** (*white cap*). Contains human serum.
 - 1 x 1.5 ml** Ready to use **Calibrator***; Calibrator (*green cap*), Human serum containing ANA. Concentrations in EU/ml are printed on the label.
 - 1 x 12 ml** Ready to use **anti-human IgG Conjugate***. Contains 0.05% Proclin 300. Color coded pink.
 - 1 x 60 ml** Ready to use **Serum Diluent***. Color coded blue.
 - 1 x 12 ml** Ready to use **TMB Enzyme Substrate. Protect from light.**
 - 1 x 12 ml** Ready to use **Stop Solution**. Contains 0.5M H₂SO₄.
 - 2 vials** Powder **Wash Buffer**. Reconstitute to one liter each.
 - 2 x** Protocol Sheets
- *Contains <0.1% NaN₃

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Materials Required But Not Provided

- Deionized or distilled water
- Squeeze bottle to hold diluted wash buffer
- Pipettors capable of delivering 5 µl to 1000 µl
- Disposable pipette tips
- Clean test tubes 12 x 75 mm and test tube rack
- Timer
- Absorbent paper
- Microplate reader capable of reading absorbance values at 450nm. If dual wavelength microplate reader is available, the reference filter should be set at 600-650 nm.
- Automatic microplate washer capable of dispensing 200 µl

SPECIMEN COLLECTION AND HANDLING

Only serum specimens should be used in this procedure. Grossly hemolyzed, lipemic or microbially contaminated specimens may interfere with the performance of the test and should not be used. Store specimens at 2-8°C for no longer than one week. For longer storage, serum specimens should be frozen. Avoid repeated freeze and thaw of samples.

PROCEDURE

Procedural Notes

- Before starting with the assay read carefully the product insert.
- Let serum specimens and test reagents equilibrate at room temperature for at least 30 minutes before starting with the test procedure. Return all unused specimens and reagents to refrigerator immediately after use.
- All dilutions of the patient samples should be prepared prior to starting with the assay.
- Good washing technique is critical. If washing is performed manually, adequate washing is accomplished by directing a forceful stream of wash buffer with a wide tip wash bottle across the entire microplate. **An automated microplate washer is recommended.**
- Use a multichannel pipette capable of delivering 8 wells simultaneously. This speeds the process and provides for a more uniform incubation time.
- For all steps, careful control of timing is important. The start of all incubation periods begins with the completion of reagent addition.
- Addition of all samples and reagents should be performed at the same rate and in the same sequence.
- Remove required microwell strips from the pouch and carefully reseal the pouch to prevent condensation in the unused wells. Return pouch immediately to refrigerator.

Test Method

- Step 1** Let all reagents and specimens equilibrate at room temperature.
- Step 2** Label protocol sheet to indicate sample placement in the wells. It is good laboratory practice to run samples in duplicate.
- Step 3** Consult the specimen layout (page 4) for a proper distribution of specimens and reagents.
- Step 4** Prepare a **1:101** dilution of the patient samples by mixing **5 µl** of the patient sera with **500 µl** of Serum Diluent.

SEMI-QUANTITATIVE

A	BLANK	S5		
B	NEG	S6		
C	POS	S7		
D	CAL	S8		
E	S1	S9		
F	S2	S10		
G	S3	S11		
H	S4	S12		
	1	2	3	4

Specimen Layout

Step 5 Pipette **100 µl** of Ready to Use Calibrator, Positive and Negative controls and diluted patient samples to the appropriate microwells as per protocol sheet.

Note: Include one well which contains **100 µl** of the Serum Diluent as a reagent blank. Zero the ELISA reader against the reagent blank. The absorbance of the reagent blank should not be more than 0.3 when read against air.

Step 6 Incubate **30 minutes** (± 5 min) at room temperature.

Step 7 Wash **4x** with wash buffer. For manual washing, fill each microwell with reconstituted wash buffer. Discard the fluid by inverting and tapping out the contents of each well or by aspirating the liquid from each well. To blot at the end of the last wash, invert strips and tap the wells vigorously on absorbent paper towels. For automatic washers, program the washer as per manufacturer's instructions.

Step 8 Pipette **100 µl** of Conjugate into microwells.

Step 9 Incubate **30 minutes** (± 5 min) at room temperature.

Step 10 Wash all microwells as in Step 7.

Step 11 Pipette **100 µl** of Enzyme Substrate into each microwell in the same order and timing as for the Conjugate.

Step 12 Incubate **microwells 30 minutes** (± 5 min) at room temperature.

Step 13 Pipette **100 µl** of Stop Solution into each microwell using the same order and timing as for the addition of the Enzyme Substrate. Read absorbance within 1 hour of adding Stop Solution.

Step 14 Read absorbance of each microwell at **450 nm** using a single wavelength microplate reader set at zero absorbance. If a dual wavelength is used, set the reference filter to 620 nm.

	Anti-ANA	anti-hu tTG	
	Abs. conc. added (EU/ml)	Abs. conc. obtained (EU/ml)	% Recovery
Sample 1	91.6	82.2	111.4
Sample 2	93.5	93.4	100.1
Sample 3	66.9	68.0	98.4

Quality Control

Calibrators, Positive and Negative Controls and a reagent blank must be run with each assay to verify the integrity and accuracy of the test. The absorbance reading of the reagent blank should be <0.3. The negative control must be <20 EU/ml. If the test is run in duplicate, take the mean of the two readings to determine the concentration of ANA. We recommend borderline samples be tested with a fresh sample taken at a later date to ensure accuracy.

RESULTS

Calculations

Concentrations of the patient samples can be determined as follows:

QUALITATIVE DETERMINATION

Results obtained by this method should be reported as positive or negative.

$$\frac{\text{Abs. (OD) of Test Sample}}{\text{Abs. (OD) of Calibrator}} \times \text{EU/ml of Calibrator} = \text{EU/ml Test Sample}$$

Interpretation

The following serves only as a guide in the interpretation of the laboratory results. These values were determined by testing 64 adult normal blood donors. The values depicted below are the mean of the normal subjects plus 3SD. Each laboratory must determine its own normal values.

ANA value	Interpretation
<20 EU/ml	Negative
20-25 EU/ml	Indeterminate (Borderline)
>25 EU/ml	Positive

LIMITATIONS OF THE PROCEDURE

ANA should not be performed on grossly hemolyzed, microbially contaminated or lipemic samples. The method should be used for testing human serum samples only. Results obtained serve only as an aid in the diagnosis and should not be interpreted as diagnostic in themselves. Some patients with some of the connective tissue disorders may be ANA negative. Similarly as the ANA occur in other than connective tissue disorders and hence the presence of ANA must be interpreted in light of clinical and other laboratory findings which may include antigen specific tests such as to RNP, Sm, SS-A (Ro), SS-B(La) and other nuclear and cytoplasmic antigens.

EXPECTED VALUES

The expected values in a normal population are negative (<20 EU/ml for adults and children). However, it has been determined that some apparently healthy, asymptomatic individuals may test positive for ANA.

Anti-Nuclear Antibodies (ANA) and Disease Association

Disease	% Incidence
SLE	95-100
Scleroderma	60-90
MCTD	100
Sjogren's syndrome	40-70
Poly/Dermato-myositis	30-80
Juvenile arthritis	20-50
Raynaud's	20-60
Rheumatoid arthritis	30-50
Infectious disease	?
Thyroid disease	30-50
Fibromyalgia	15-25
Normal subjects	~10

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PERFORMANCE CHARACTERISTICS

The utility of the Immulisa™ ANA ELISA was determined by comparing the results with:

- another commercially available ANA ELISA method and
- ANA immunofluorescence method on Hep2.

Normal Range: The normal range was established by testing 64 serum samples from apparently healthy donors obtained from the Red Cross. The mean plus three standard deviations of the mean of this normal population was used to determine the cut-off between normal and borderline positive individuals.

Comparative Specificity and Sensitivity

A Immulisa™ ANA ELISA vs. another commercial ANA ELISA Method: A total of 66 samples were tested on the Immulisa™ ANA kit and another commercially available FDA approved ANA ELISA kit. The results of these studies follow:

	Other ELISA		
	Positive	Negative	Total
Immulisa™ ANA ELISA			
Positive	41	8	49
Negative	5	12	17
Total	46	20	66

Relative Agreement: 80%
Relative Sensitivity: 89%
Relative Specificity: 60%

- B. To ensure the reliability of Immulisa™ ANA In detecting ANA , sera were also tested on HEp2, the substrate of choice of detecting ANA by immunofluorescence. Immulisa™ ANA ELISA vs. Immulisa™ ANA on HEp2: A total of 292 samples were tested for ANA and the results are summarized below:

		ANA HEp2		Total
		Positive	Negative	
Immulisa™ ANA	Positive	123	6	129
	Negative	7	156	163
	Total	130	162	292

Relative Agreement: 96%
Relative Sensitivity: 95%
Relative Specificity: 96%

- C. Cross Reactivity: A total of 55 disease controls from other autoimmune diseases usually known to be ANA negative such as pemphigus were tested. Only four were tested positive which is about the same number reported to be positive in normal subjects. Results of this study appear below

Disease	Number Tested	Number Positive	% Positive
Celiac Disease	19	1	5%
Dermatitis Herpetiformis	16	2	12%
Pemphigus	20	1	5%

Precision:

Based on 10 replicates, the intra-assay and inter-assay Coefficient of Variation (CV) of the ANA ELISA test were calculated.

	Inter-assay		Intra-assay	
	EU/ml	CV	EU/ml	CV
High	107.7	6.4%	125.9	6.8%
Medium	51.4	6.2%	52.7	7.0%
Low	19.5	5.2%	20.3	8.1%

Recovery:

Samples with known ANA antibody concentrations were mixed with appropriate dilutions of another positive sample with known amounts of ANA antibody. ANA levels of the mixed samples were determined and from the values obtained the percent recovery calculated. The results are as follows: